HDL-3 is a Superior Predictor of Carotid Artery Disease in a Case-Control Cohort of 1725 Participants

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Background—Recent data suggest that high-density lipoprotein cholesterol (HDL-C) levels are likely not in the causative pathway of atheroprotection, shifting focus from HDL-C to its subfractions and associated proteins. This study’s goal was to determine which HDL phenotype was the better predictor of carotid artery disease (CAAD).

Methods and Results—HDL-2 and HDL-3 were measured in 1725 participants of European ancestry in a prevalent case-control cohort study of CAAD. Stratified analyses were conducted for men (n=1201) and women (n=524). Stepwise linear regression was used to determine whether HDL-C, HDL-2, HDL-3, or apolipoprotein A1 was the best predictor of CAAD, while adjusting for the confounders of censored age, diabetes, and current smoking status. In both men and women, HDL-3 was negatively associated with CAAD (P=0.0011 and 0.033 for men and women, respectively); once HDL-3 was included in the model, no other HDL phenotype was significantly associated with CAAD. Addition of paraoxonase 1 activity to the aforementioned regression model showed a significant and independent (of HDL-3) association with CAAD in men (P=0.001) but not in the smaller female subgroup.

Conclusions—This study is the first to contrast the associations of HDL-2 and HDL-3 with CAAD. We found that HDL-3 levels were more predictive of CAAD status than HDL-2, HDL-C, or apolipoprotein A1. In addition, for men, paraoxonase 1 activity improved the overall model prediction for CAAD independently and additively with HDL-3 levels. Further investigation into the molecular mechanisms through which HDL-3 is associated with protection from CAAD is warranted. (J Am Heart Assoc. 2014;3:e000902 doi: 10.1161/JAHA.114.000902)

Key Words: atherosclerosis • carotid arteries • high-density lipoprotein • lipids • lipoproteins
had significantly higher levels of HDL-C; however, the LIPA<sub>A</sub> single nucleotide polymorphism was not associated with risk of myocardial infarction (P=0.85). Together, the failure of these 2 studies to establish a causal link between HDL-C level and cardiovascular disease has raised new doubts regarding the cardioprotective nature of HDL.

More recent evidence from the Multi-Ethnic Study of Atherosclerosis (MESA) has revealed a possible explanation for the negative results of HDL-C and cardiovascular events. In this study, Mackey et al evaluated the association of HDL-C and HDL particle concentration measured by nuclear magnetic resonance spectroscopy (HDL-P) with incident CHD (n=227 events). As expected, HDL-C and HDL-P were highly correlated (Spearman’s correlation coefficient of 0.69, P<0.001); however, in multivariate regression models, HDL-P was the superior predictor of incident CHD in comparison to HDL-C. This finding indicated that although HDL-C captured a large portion of HDL-P variation, HDL-C measurements alone did not reflect the individual elements of HDL captured by HDL-P that were primarily responsible for cardioprotection.

Aspects of HDL that are not captured by HDL-C include its immensely complex proteome, with recent estimates of 64 associated proteins. Broadly, HDL is composed of 2 subspecies, HDL-2 and HDL-3, that can be separated by ultracentrifugation and electrophoresis. Both HDL-2 and HDL-3 have distinct biochemical, physiologic, and metabolic functions. Investigations contrasting the cardioprotection of HDL-2 or HDL-3 have yielded conflicting results, with aspects of both subspecies being associated with decreased risk of CVD.

Given the recent evidence suggesting that HDL-C level does not have a direct causal role in cardioprotection, we hypothesized that specific aspects of HDL not completely correlated with HDL-C level were superior predictors of CAAD, as defined by >50% stenosis in either carotid artery. Specifically, we sought to determine whether the level of one of the 2 subspecies of HDL (HDL-2 versus HDL-3) was a superior predictor of CAAD status in a prevalent case-control cohort from the Carotid Lesion Epidemiology and Risk (CLEAR) study, a Seattle, Washington-based repository composed primarily of veterans that was collected to identify risk factors for CAAD, CAAD progression, and other atherosclerotic disease end points. In addition, we attempted to elucidate whether functional aspects of HDL, such as its associated proteins apolipoprotein A1 (apoA1) and paraoxonase 1 (PON1), independently predicted further CAAD status.

Methods

Ethics Statement

Institutional review boards at the University of Washington, Virginia Mason Medical Center, and Veterans Affairs Puget

Sample

The CLEAR study is a Seattle-based prevalent CAAD case-control study composed primarily of veterans, with controls matched by age at diagnosis (for CAAD cases) and current unaffected age (for controls). Exclusion criteria included familial hypercholesterolemia, total fasting cholesterol >400 mg/dL, hypocoagulable state and/or the use of anticoagulant medication, previous organ transplant, or the inability to provide consent. All participants underwent ultrasound assessment of their carotid arteries for the presence or absence of atherosclerotic plaque except for a small number of participants with CAAD that had a prior carotid endarterectomy for symptomatic obstruction. CAAD case status was defined as >50% stenosis in either carotid artery as determined by ultrasound, whereas controls were also imaged and had <15% stenosis in both carotid arteries and absence of peripheral artery disease or CHD. The cohort consisted of 688 CAAD cases and 1037 controls. The few participants with moderate carotid stenosis (15% to 49% obstruction in at least one carotid artery) were excluded from analysis. Censored age was the age at CAAD diagnosis for cases and age at enrollment and blood draw for controls. Current smoking status was obtained by self-report. Insulin use was determined by self-report matched to hospital pharmacy records.

The study population for this analysis consisted of 1725 European-ancestry participants from the previously described CLEAR study. To avoid population stratification, smaller numbers of participants with non-European ancestry were excluded from all analyses presented in this paper. European genetic ancestry was confirmed by principal components analysis using STRUCTURE and single nucleotide polymorphisms from the Illumina CVD chip or 550k BeadChip data. Descriptive statistics of the cohort are presented in Table 1.

Lipid Measurements

Standard methods were used to determine total cholesterol, triglycerides, and HDL in fasting whole plasma using an Abbott Spectrum analyzer. HDL fractions 2 and 3 were determined by precipitating HDL-2 from isolated total HDL, measuring HDL-3 in the supernatant, and subtracting this from total HDL to obtain HDL-2. ApoA1 was measured as previously described. PON1 activity was measured by the rate of enzymatic degradation of phenylacetate (AREase) by a continuous spectrophotometric assay with lithium heparin plasma, as AREase is least affected by the functional PON1<sub>A</sub> polymorphism and also is more closely related...
to PON1 protein levels.\textsuperscript{25,26} PON1 AREase activity was measured in triplicate and averaged. All lipid and associated protein measurements had approximate standard distributions. All data were generated blinded to CAAD status.

**Statistical Analyses**

Analyses were done in R (http://www.r-project.org/) using the available standard regression tools. All participants had complete phenotype and covariate data for regression analyses. Because there is a known sex-dependent difference in HDL levels, we chose to perform sex-stratified analyses.

Sex-specific analyses comparing risk factors between CAAD cases and controls used either the Wilcoxon rank sum test (for continuous variables) or Pearson’s chi-square test (for categorical variables) to determine significance. Correlation of covariates was summarized using Pearson’s pairwise correlation coefficient.

Given the high correlation between the measurements, we performed stepwise logistic regression on the phenotype of CAAD, with HDL-C, HDL-2, HDL-3, and apoA1 entering the model. Model comparison was done using Akaike’s information criterion, beginning with a base model composed of the known confounders of CAAD status: censored age, diabetes status, and current smoking status. The measurement that best improved model prediction of CAAD via Akaike’s information criterion was retained in the final model. A secondary analysis considered the addition of PON1 AREase activity to HDL-3 levels and performed stepwise logistic regression, also considering censored age, current smoking, and diabetes status, to determine whether PON1 AREase activity predicted additional CAAD variance independent of the other lipid measurements.

Statin drug use can also affect HDL levels; however, because CAAD is treated with statins, statin use was confounded for CAAD status and could not be included in a model predicting CAAD. We noted the increase in HDL levels in the CLEAR data was not statistically significant in 57 male CLEAR participants with repeat lipid measurements before and after statin initiation. Although HDL levels do increase with statin use, the increase was not statistically significant (HDL-C, \(P=0.14\); HDL-2, \(P=0.57\); HDL-3, \(P=0.27\); Table 2).

**Results**

Demographic, clinical, and lipid variables of the studied European-ancestry subset of the CLEAR cohort are presented

### Table 1. Baseline Characteristics of the Studied European-Ancestry Subset of CLEAR, Stratified by Sex

<table>
<thead>
<tr>
<th></th>
<th>Women (N=524)</th>
<th>Men (N=1201)</th>
<th>Combined (N=1725)</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoA1, IU</td>
<td>166±28</td>
<td>138±26</td>
<td>146±29</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>63±18</td>
<td>47±15</td>
<td>52±18</td>
</tr>
<tr>
<td>HDL-2, mg/dL</td>
<td>14.7±7.8</td>
<td>8.5±5.3</td>
<td>10.4±6.8</td>
</tr>
<tr>
<td>HDL-3, mg/dL</td>
<td>49±11</td>
<td>39±11</td>
<td>42±12</td>
</tr>
<tr>
<td>PON1 AREase activity, IU</td>
<td>167±58</td>
<td>132±49</td>
<td>143±55</td>
</tr>
<tr>
<td>Current smoker</td>
<td>6% (32)</td>
<td>17% (199)</td>
<td>13% (231)</td>
</tr>
<tr>
<td>Statin use</td>
<td>24% (124)</td>
<td>40% (482)</td>
<td>35% (606)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>9% (46)</td>
<td>22% (268)</td>
<td>18% (314)</td>
</tr>
<tr>
<td>Censored age, years*</td>
<td>63.6±9.8</td>
<td>68.0±9.4</td>
<td>66.6±9.7</td>
</tr>
<tr>
<td>CAAD status</td>
<td>19% (102)</td>
<td>49% (586)</td>
<td>40% (688)</td>
</tr>
</tbody>
</table>

Mean±1 SD. Numbers after percents are counts. apoA1 indicates apolipoprotein A1; AREase, PON1 arylester hydrolysis rate; CAAD, carotid artery disease; CLEAR, Carotid Lesion Epidemiology and Risk; HDL-C, high-density lipoprotein cholesterol.

*Censored age defined as the age at CAAD diagnosis (for CAAD cases) or the age at enrollment of controls.

### Table 2. Effect of Statin Use on HDL-C, HDL-2, and HDL-3 Concentration in a Male-Only Subset of CLEAR With Repeat Lipid Measures Before and After Statin Use (n=57)

<table>
<thead>
<tr>
<th></th>
<th>Before Statin</th>
<th>After Statin</th>
<th>(P) Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL-C</td>
<td>42.63±13.03</td>
<td>46.44±13.78</td>
<td>0.14</td>
</tr>
<tr>
<td>HDL-2</td>
<td>6.91±3.99</td>
<td>7.28±4.23</td>
<td>0.57</td>
</tr>
<tr>
<td>HDL-3</td>
<td>35.72±9.47</td>
<td>37.38±10.51</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Mean±1 SD. HDL indicates high-density lipoprotein cholesterol.

*Tests used: 2-sample, 2-sided t test without the assumption of equal variance.
in Table 1. The cohort was composed of 1201 men and 524 women, of which 586 men (49%) and 102 women (19%) had CAAD. Women had lower rates of smoking (6% versus 19%), statin use (24% versus 40%), and diabetes (9% versus 22%). In addition, women had higher levels of all HDL phenotypes (HDL-C, HDL-2, HDL-3, apoA1 and PON1). Due to these sex-dependent differences in lipid and clinical covariates, stratified analyses were conducted to determine which HDL measure was the better predictor of CAAD.

There were significant (P<0.05) differences for all clinical and lipid covariates between CAAD participants and controls for both men (Table 3) and women (Table 4). Those with CAAD had significantly lower levels of apoA1, HDL-C, HDL-2, HDL-3, and PON1 AREase activity compared with controls for both men and women and also had significantly higher rates of current smoking and diabetes. In addition, censored age was higher for both male and female participants with CAAD when compared with controls.

The pairwise correlation between each of the studied clinical and lipid covariates was considerable (Figure 1). ApoA1, HDL-C, HDL-2, and HDL-3 were all strongly and positively correlated with each other (pairwise correlation coefficients, r≥0.75). PON1 AREase activity was also positively correlated with the other lipid measurements, albeit not as strongly (r≥0.26). Current smoking status and diabetes were each negatively correlated with each of the aforementioned lipid measurements, although they were not highly correlated with each other (r=0.04).

In men, HDL-3 was the lipid measurement that explained the greatest amount of CAAD variation (1.6%) and was
negatively associated with CAAD (OR = 0.97 [95% CI: 0.95 to 0.98], P = 0.00011; Table 5). In addition, PON1 AREase activity was negatively associated with and improved model prediction of CAAD (0.7% of CAAD variation; OR = 0.99 [95% CI: 0.98 to 0.99], P < 0.001) in men. A 10-mg/dL increase in HDL-3 was calculated to decrease the odds of CAAD by ≈24% (OR = 0.76 [95% CI: 0.58 to 0.99]). Although underpowered, post hoc analysis found no evidence of interaction (P > 0.05) between either HDL-3 levels or PON1 AREase activity with any demographic covariate (censored age) or clinical covariate (smoking and diabetes status).

**Discussion**

In the current study, we have used a CAAD case-control cohort of participants with European ancestry to evaluate the effects of apoA1, HDL-C, HDL-2 HDL-3, and PON1 AREase activity on CAAD risk separately in men (n = 1201) and women (n = 524). Using stepwise regression to find the best predictor of CAAD from the highly correlated lipid measurements of apoA1, HDL-C, HDL-2, and HDL-3, we have identified HDL-3 as the HDL phenotype that captures the most CAAD status variance in both men and women. With HDL-3 in the regression model, none of the remaining HDL-related measurements, except PON1 AREase level, improved the model for CAAD. We noted no potential confounding of age on HDL lipoprotein levels that could negate our findings. PON1 AREase activity had a significant impact on CAAD risk that was independent of HDL-3 levels in men only. To the best of our knowledge, this represents the first CAAD case-control study with population-based controls to evaluate the effects of HDL subspecies on CAAD risk.

Prior work on the associations of HDL-2 and HDL-3 with CVD has yielded conflicting results. In a recent literature review, it was reported that 45% of 37 total case-control studies found a statistically significant decrease in CVD cases for both HDL-2 and HDL-3 levels, 26% found a significant decrease in HDL-2 only, 11% found a significant decrease in HDL-3 only, and 17% found no statistically significant decrease in either HDL subfraction.27 The vast majority of these studies collected CHD cases; to the best of our knowledge, only one prior study has looked at CAAD: Atger et al examined 181 asymptomatic hypercholesterolemic men

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**Figure 1.** Correlation matrix for plasma lipid measurements. Values inside each box represent r, the pairwise correlation coefficient, unadjusted for covariates. apoA1 indicates apolipoprotein A1; HDL-C, high-density lipoprotein cholesterol; PON1, paraoxonase 1.

**Table 5.** Best-Fit Model From Stepwise Linear Regression Predicting CAAD Status in Men Using Lipid and Clinical Covariates (N = 1201)

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Odds Ratio (95% CI)</th>
<th>% CAAD Variation</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.0055 (0.0013 to 0.022)</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Censored age, years*</td>
<td>1.10 (1.09 to 1.13)</td>
<td>11.18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current smoker</td>
<td>5.63 (3.74 to 8.62)</td>
<td>7.31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetic</td>
<td>3.41 (2.38 to 4.95)</td>
<td>4.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-3, mg/dL</td>
<td>0.97 (0.95 to 0.98)</td>
<td>1.59</td>
<td>0.00011</td>
</tr>
<tr>
<td>PON1 AREase activity, IU</td>
<td>0.99 (0.98 to 0.99)</td>
<td>0.68</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Mean±1 SD. AREase indicates PON1 arylester hydrolysis rate; CAAD, carotid artery disease; HDL, high-density lipoprotein.

*Censored age defined as the age at CAAD diagnosis (for CAAD cases) or the age at enrollment of controls.
Table 6. Best-Fit Model From Stepwise Linear Regression Predicting CAAD Status in Women Using Lipid and Clinical Covariates (N=524)

<table>
<thead>
<tr>
<th>Odds Ratio (95% CI)</th>
<th>% CAAD Variation</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.00063 (0.00005 to 0.007)</td>
<td>—</td>
</tr>
<tr>
<td>Censored age, years*</td>
<td>1.11 (1.08 to 1.15)</td>
<td>12.62</td>
</tr>
<tr>
<td>Current smoker</td>
<td>3.56 (1.48 to 8.31)</td>
<td>5.05</td>
</tr>
<tr>
<td>Diabetic</td>
<td>3.89 (1.82 to 8.35)</td>
<td>1.51</td>
</tr>
<tr>
<td>HDL-3, mg/dL</td>
<td>0.97 (0.95 to 0.99)</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Mean±1 SD. CAAD indicates carotid artery disease; CI, confidence interval; HDL, high-density lipoprotein.

*Censored age defined as the age at CAAD diagnosis (for CAAD cases) or the age at enrollment of controls; HDL, high-density lipoprotein.

by ultrasound to determine the presence of CAAD, in addition to femoral and abdominal aorta stenosis. No significant difference in either HDL-2 and HDL-3 levels was found between 43 men with CAAD compared with the 138 other men.28 We note, however, that their case sample size was only 7% of our size (586 in our study versus 43 in their study); thus, they lacked power to detect the effects of HDL-3 that we identified.

The majority of prior work on the subject of HDL subfractions and CVD was done in studies of CHD and found a stronger association of HDL-2 with cardioprotection.27,29–32 One hypothesis for this correlation related to the much higher density of apoA1 on the larger HDL-2 molecule compared with HDL-3. We evaluated this hypothesis in the current study through inclusion of apoA1 as one of the possible HDL-related covariates in the stepwise regression model; however in our analyses, HDL-3 was the best predictor of CAAD variance, and with HDL-3 in the regression model, none of the other HDL-related covariates (HDL-C, HDL-2, or apoA1) were able to improve prediction of CAAD status. We note that HDL-3 is associated with other apolipoproteins, namely, A2, A3, A4, and pre-B1.13 Because these were not measured in the current study, we were unable to evaluate their individual effects on CAAD risk.

HDL-3 is the smaller, denser, and more lipid-poor of the 2 subfractions of HDL. HDL-3 is strongly antioxidant, and in prior work, it has been demonstrated that the antioxidant capability of HDL increases with density.33 In addition, HDL-3 is closely associated with the glycoprotein enzyme PON1.34 PON1 is itself atheroprotective35–37 and can prevent low-density lipoprotein oxidation38,39 and HDL oxidation40 (other functions of PON1 are summarized in a recent review article41). To address the possibility that our association of HDL-3 with CAAD was due to the effects of its association with PON1, we included PON1 AREase activity as a covariate in the stepwise regression model. Interestingly, in men, both HDL-3 and PON1 AREase activity were retained in the final model, suggesting that the atheroprotective effects of HDL-3 are independent and additive of PON1 enzyme activity. As noted previously, the molecular mechanisms for this association of HDL-3 with CAAD could be due to unmeasured apolipoproteins with which HDL-3 is correlated. It is notable that HDL-3 likely has antioxidant properties that are independent of PON1.33 The lack of detection of an additional PON1 effect in women may have been due to insufficient statistical power in that smaller group.

Our finding of HDL-3 being the best predictor of CAAD status is incongruent with many studies that have used similar multivariate regression methods and found HDL-2 to be more significantly associated; however, as noted above, the majority of past work regarding HDL-2 and HDL-3 has focused on CHD rather than CAAD. Although both disease states are driven by atherosclerosis, the underlying pathogenesis is likely different,42 as shown by the divergence of genes that are associated with myocardial infarction43,44 versus ischemic stroke45,46 or carotid intimal media thickening47 (no genomewide association study has been performed for CAAD to date). In this context, our finding of HDL-3 being most strongly associated, independent of PON1, may represent further evidence that the pathology of CAAD is distinct.

Several limitations of the current study must be considered. First, this cohort was composed only of participants of European ancestry, limiting inferences from our data to participants of other races. Second, men composed the majority of both the cohort and the CAAD cases (102 women versus 586 men), leaving our female-only analyses statistically less powered. Third, due to confounding with CAAD status, our analyses could not adjust for the effects of statin use on HDL-C, HDL-2, and HDL-3 levels. Although underpowered, there was no statistically significant evidence for an increase in HDL levels in 57 male participants in pre– and post–statin-initiation data. Similarly, data from the COMparative Effects on Lipid Levels of Niaspan and a Statin versus Other Lipid-Modifying Therapies (COMPELL) study, which studied the effects of 4 different statins on lipid profiles in 292 participants (50% female), did not show a statistically significant increase in HDL-3 levels at 8- or 12-week follow-up.48 Regardless, the excess of
statin use in cases would result in more conservative testing of our hypothesis of HDL phenotype differences between CAAD cases and controls if statin use does increase HDL levels, as found elsewhere.49

In conclusion, our analysis of HDL subfraction data and CAAD has found that, of the subphenotypes apoA1, HDL-C, HDL-2, and HDL-3, only HDL-3 best predicts CAAD risk and the remaining phenotypes do not add significant predictive power. The effects of HDL-3 were independent of and additive with PON1 enzyme activity. Given the importance of CAAD as a risk factor for ischemic stroke and as a marker of CVD, further work elucidating the molecular mechanisms through which HDL-3 is cardioprotective for CAAD is warranted.

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Disclosures
None.

References


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